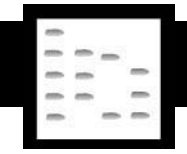
PEPTIDES AND PROTEINS



Liquid Chromatography

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Introduction

High performance liquid chromatography (HPLC) has proved extremely versatile in aiding the isolation of peptides from a wide variety of sources, including complex proteolytic and/or chemical cleavage mixtures of proteins as well as crude mixtures arising from the ever-increasing employment of solid-phase peptide synthesis. The complexity of such peptide mixtures may vary considerably depending on the source. Thus, for instance, peptides obtained from biological tissues are often found in very small quantities and may require extensive purification, while impurities arising from peptide synthesis are usually closely related to the peptide of interest (deletion, terminated or chemically modified peptides), missing perhaps only one amino acid residue, and may be difficult to separate. The development of high performance separation techniques has enabled the much more efficient utilization of peptide size (size exclusion chromatography or SEC) and net charge (ion exchange chromatography or IEC) compared to the much inferior and slower separations achievable by classical SEC and IEC. In addition, reversed-phase chromatography (RPC), a technique that does not have a classical predecessor, has proved to be an excellent means of analysing peptides based on their relative hydrophobic/hydrophilic characteristics.

With the requirement for efficient separation protocols constantly fueling demands for improvements in HPLC instrumentation and packings, it is perhaps not surprising that researchers have generally overlooked the potentially useful mixed-mode properties of high performance packings which, with careful manipulation of mobile-phase conditions, can offer a novel addition to the HPLC arsenal. In our experience, all HPLC packings have exhibited some deviation from ideal solute retention behaviour; thus, SEC packings often exhibit some hydrophobic and ionic characteristics, IEC packings exhibit hydrophobic character to a greater or lesser extent and RPC packings frequently also exhibit some ion exchange properties. Mobile phases may then be designed to minimize or eliminate such mixed-mode characteristics which, if left unchecked, can lead to poor resolution and broad, tailing peaks. The mixed hydrophobic and charged characteristics of peptides make these molecules particularly susceptible to such nonideal retention behaviour. However, the reverse is that certain so-called nonideal packing characteristics – in the sense that they represent an unintended, nonspecific property of a column packing – may significantly enhance the resolving power of that packing.

As noted above, the hydrophobic/hydrophilic characteristics of peptides, a result of the nonpolar/polar nature of the side chains making up the peptide, are well recognized, particularly in terms of peptide elution from a reversed-phase column in order of increasing peptide hydrophobicity. What has not been so well recognized until relatively recently, however, is that since ion exchange stationary phases are designed to be as hydrophilic (or as neutral) as possible to avoid hydrophobic interactions, such hydrophilic characteristics may be used to advantage if harnessed properly. The present article describes, and illustrates with practical examples, how the ionic nature of a strong cation exchange matrix may be overlayed with hydrophilic characteristics, thus effecting peptide separations by a combined hydrophilic/cation exchange mechanism. This novel mixed-mode approach, termed hydrophilic interaction–cation exchange chromatography (HILIC-CXC) offers drastically different selectivity to RPC, underlining its value as a complementary approach.

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General Principles and Conditions of HILIC-CXC

The term hydrophilic interaction chromatography (HILIC) was originally coined to describe separations based on solute hydrophilicity. Thus, separation by HILIC, in a manner similar to normal-phase chromatography (to which it is related), depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase, i.e. solutes are eluted in order of increasing overall hydrophilicity (decreasing hydrophobicity). Characteristic of HILIC-CXC separations is the presence of a high initial organic

modifier (e.g. acetonitrile) concentration in the mobile phase which, concomitant with overcoming any undesirable hydrophobic column behaviour, serves to promote hydrophilic interactions between the solute and the stationary phase, specifically a strong cation exchange stationary phase with significant hydrophilic characteristics. Such characteristics generally only become apparent once any matrix hydrophobicity has been suppressed. Hydrophilic interactions between peptides and the cation exchange packing are then overlayed on the ionic interactions between basic (potentially positively charged) peptides and the negatively charged packing.

Different ion exchange matrices exhibit differing degrees of hydrophobic characteristics. In order to gain the full benefit of the HILIC mode, it is important to overcome unwanted hydrophobic properties of the matrix with as low a level of organic modifier as possible – the ion exchange matrix should be as hydrophilic as possible. In this way, there is a greater organic modifier range open to the researcher to effect mixed-mode HILIC-CXC peptide separations. In our hands, the PolySulfoethyl A strong cation exchange column (based on a polypeptide coating, poly[2-sulfoethylaspartamide], covalently bonded to silica) has proven to be very hydrophilic, hence its use in the separations shown in the current article.

Typical conditions for mixed-mode HILIC-CXC are a linear increasing sodium perchlorate ($NaClO_4$) gradient (2–20 mmol L^{-1} $NaClO_4$ min^{-1}) at pH 3–7, with the mobile phase containing 15–80% acetonitrile (ACN). Thus, the cation exchange column separates peptides based on net positive charge and this separation mode is then complemented by the presence of ACN overcoming undesirable hydrophobic interactions and promoting desirable hydrophilic interactions. $NaClO_4$ is suitable for this mixed-mode approach due to its excellent solubility in aqueous solution even in the presence of high concentrations of organic modifier. Where the charged characteristics of the components of a specific peptide mixture are unknown, relatively low pH conditions are a good starting point in order to maximize the basic character of the peptide solutes and, hence, enhance ionic interactions with the negatively charged strong cation exchange matrix. Thus, at pH 3, any acidic (potentially negatively charged) residues (aspartic acid, glutamic acid) will be mainly in the protonated, neutral form. In addition, a full positive charge on the basic residue histidine ($pK_a = 6.5$) is also assured at low pH. Although perhaps a less obvious concern, there is also a need to be cautious with the pH of the mobile phase when considering basic residues such as lysine ($pK_a \sim 10$) and arginine ($pK_a \sim 12$). This caution arises from reduction in pK_a of such basic residues frequently observed in a nonpolar environment, represented in the present case by the nonpolar (relative to water) organic modifier ACN. Thus, due to the presence of high concentrations of ACN (up to 90%) characteristic in HILIC-CXC mobile phases, the use of relatively low pH conditions to ensure full protonation (i.e. a full positive charge) of basic side chains is a wise precaution. As an additional benefit, silica-based ion exchange columns tend to be more stable over a period of time at pH 3 compared to pH values around neutrality.

The general principles of HILIC-CXC are well demonstrated in **Figure 1** which compares the separation of four random coil peptides, denoted S2, S3, S4 and S5 (**Table 1**), by RPC (top), CXC (middle) and HILIC-CXC (bottom). This four-peptide mixture contains peptides with the same net positive charge (+2) and subtly increasing hydrophobicity (S2 < S3 < S4 < S5). Note that the only difference between the CXC and HILIC-CXC runs is the presence of 10% (v/v) ACN in the former compared to 80% (v/v) in the latter.

From **Figure 1**, peptides are as expected, eluted from the RPC column (top) in order of increasing hydrophobicity. Under characteristic cation exchange conditions (middle), the presence of 10% (v/v) ACN helps to eliminate unwanted hydrophobic interactions between solutes and the stationary phase, and the four peptides are very poorly resolved, as expected given the identical net charge on the peptides. Note, however, that the low concentration of ACN (10%) has already induced hydrophilic interactions with the matrix in that the elution order is already opposite to that of RPC (the most hydrophobic peptide is eluted first and the most hydrophilic last). In comparison, under HILIC-CXC conditions (bottom), the elution order remains the same but the peptides are now well resolved. Clearly, to effect a separation of these peptides on the cation exchange column, an increased concentration (80% (v/v)) of ACN is required in the mobile phase in order to promote hydrophilic interactions with the stationary phase to complement the ionic interactions.

Optimization of HILIC-CXC Run Conditions

While flow rates of 0.5–2.0 mL min^{-1} are generally favoured for analytical ion exchange separations and, by extension, mixed-mode HILIC-CXC runs, the effect of varying flow rates is somewhat limited. In contrast, variations in gradient rate (increasing counterion concentration per unit time) have the potential for large effects on the efficiency of separation. In addition, one option available to HILIC-

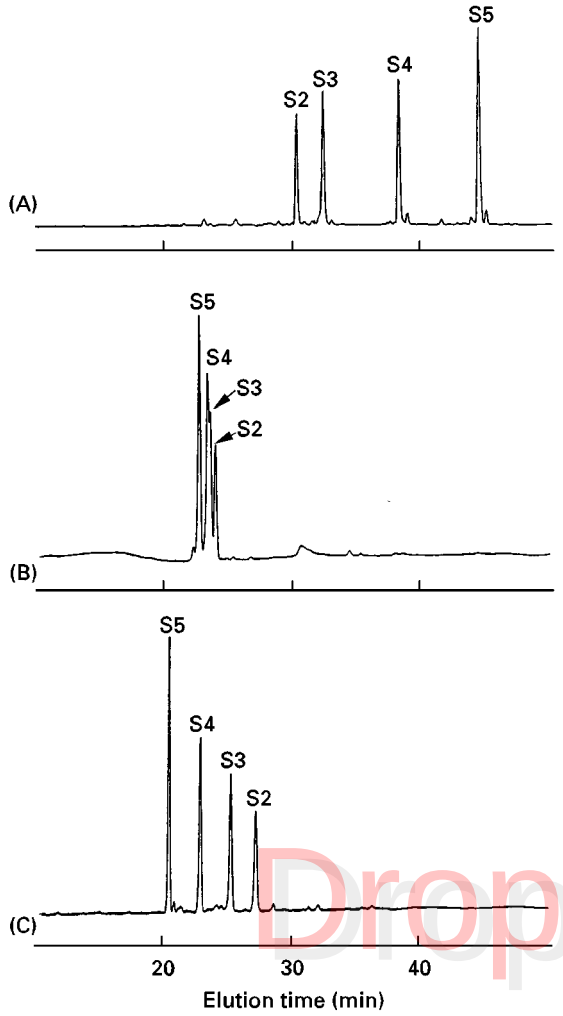

Figure 1 General principles of HILIC-CXC versus RPC: (A) RPC; (B) CXC; (C) HILIC/CXC. Columns: Zorbax SB300-C_8 reversed-phase column (150 × 4.6 mm i.d., 5 μm particle size, 30 nm pore size) from Hewlett-Packard PolySulfoethyl a strong cation exchange column (200 × 4.6 mm i.d., 5 μm, 30 nm) from PolyLC Conditions: RPC, linear AB gradient (0.5% acetonitrile min^{-1}) at a flow rate of 1 ml min^{-1}, where eluent A is 20 mmol L^{-1} aq. triethylammonium phosphate (TEAP), pH 3, and eluent B is eluent A containing 60% (v/v) acetonitrile, both eluents containing 100 mmol L^{-1} $NaClO_4$; CXC, linear AB gradient 5 mmol L^{-1} $NaClO_4$/min^{-1}, following 5 min isocratic elution with eluent A at a flow rate of 1 ml min^{-1}, where eluent A is 20 mmol L^{-1} aq. TEAP, pH 3, containing 10% (v/v) acetonitrile and eluent B is eluent A containing 400 mmol L^{-1} $NaClO_4$. HILIC/CXC, same conditions as for CXC, except for 80% (v/v) acetonitrile in eluents A and B; all runs carried out at 30°C and peaks detected by absorbance at 210 nm. (Reproduced with permission from Mant *et al.*, 1998a.)

CXC, but not to separations based solely on an ion exchange mechanism, is to vary the ACN concentration in the mobile phase in order to modulate the magnitude of hydrophilic interactions overlaying the ion exchange process.

Variation of Salt Gradient Rate

Figure 2 shows the effect of varying gradient rate of $NaClO_4$ (under conditions of a consistent and high ACN concentration) on the HILIC-CXC elution profile of a mixture of six cyclic, amphipathic β-sheet peptides. These peptides offer a particularly stringent test of the capabilities of the HILIC-CXC approach in that only the two residues making up the hydrophilic face (the face binding preferentially to the ion exchange matrix) of the β-sheet are varied (Table 1): the overall net charge of all the peptides is identical (+2); note that the hydrophobic face of all six peptide analogues, made up of leucine and valine residues (**Table 1**), is constant. From **Figure 2**, reducing the gradient rate from 5 mmol L^{-1} $NaClO_4$ min^{-1} to 2.5 mmol L^{-1} min^{-1} and, finally, to 1 mmol L^{-1} min^{-1} clearly affects the efficiency of the separation with, interestingly, optimal separation in this mixed mode being achieved at the intermediate rate of 2.5 mmol L^{-1} min^{-1}. Thus, while reducing the gradient from 5 mmol L^{-1} min^{-1} (top) to 2.5 mmol L^{-1} min^{-1} (middle) achieved an expected improvement in resolution (peptides Dap and Arg are co-eluted at the higher gradient rate), further reduction to 1 mmol L^{-1} min^{-1} (bottom) results in a deterioration of the separation, with peptides Orn and Dab now co-eluted. Such results, while illustrating the efficacy of varying salt gradient rate, also serve to indicate the complexity of peptide retention behaviour when responding to variations in run parameters.

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Variation of Acetonitrile Concentration

Figure 3 demonstrates the effect of ACN concentration on the elution of a mixture of 12 peptides with negligible secondary structure; the numbers denote the number of potentially positively charged groups on the peptides (**Table 1**). With 20% ACN in the mobile phase (**Figure 3**A), the overall elution order of all 12 peptides is essentially based on increasing net positive charge (+1 < +2 < +3 < +4) – ionic interactions dominate the separation process. At 50% ACN (**Figure 3**B), ionic interactions are still dominant since peptides are still generally eluted in order of increasing net positive charge. However, a hydrophilic interaction mechanism also becomes more substantial, as evidenced by the improvement in the separation of peptides of like charge. Also, note the elution of peptide 14 before the lesser charged (but more hydrophilic) b3 and a3, a clear example of how more highly charged peptides may still be eluted prior to less highly charged peptides if the latter are significantly more hydrophilic than the former. A further increase in ACN concentration to 90% (**Figure 3**C)

Table 1 Synthetic peptides used in this study

Figure	*Peptide sequence*[a]	*Peptide notation*
Random coil peptides		
1, 3, 4	Ac–**R**–G–G–G–G–L–G–L–G–**K**–amide	S2, e2
1, 3, 4	Ac–**R**–G–A–G–G–L–G–L–G–**K**–amide	S3, f3
1, 3, 4	Ac–**R**–G–V–G–G–L–G–L–G–**K**–amide	S4, g4
1	Ac–**R**–G–V–V–G–L–G–L–G–**K**–amide	S5
3, 4	Ac–**R**–G–V–Y–G–L–G–L–G–**K**–amide	h3
3, 4	NH_2–**R**–G–G–G–G–L–G–L–G–**K**–amide	a3
3, 4	NH_2–**R**–G–A–G–G–L–G–L–G–**K**–amide	b3
3, 4	NH_2–**R**–G–V–G–G–L–G–L–G–**K**–amide	c3
3, 4	NH_2–**R**–G–V–Y–G–L–G–L–G–**K**–amide	d3
3, 4	Ac–G–G–G–L–G–G–A–G–G–L–**K**–amide	i1
3, 4	Ac–**K**–Y–G–L–G–G–A–G–G–L–**K**–amide	j2
3, 4	Ac–G–G–A–L–**K**–A–L–**K**–G–L–**K**–amide	k3
3, 4	Ac–**K**–Y–A–L–**K**–A–L–**K**–G–L–**K**–amide	l4
Cyclic and constrained peptides[b]		
2, 6	cyclo(V–**H**–L–Y–P–V–**H**–L–Y–P)	His
2, 6	cyclo(V–**R**–L–Y–P–V–**R**–L–Y–P)	Arg
2, 6	cyclo(V–**K**–L–Y–P–V–**K**–L–Y–P)	Lys
2, 6	cyclo(V–**O**–L–Y–P–V–**O**–L–Y–P)	Orn
2, 6	cyclo(V–**Dap**–L–Y–P–V–**Dap**–L–Y–P)	Dap
2, 6	cyclo(V–**Dab**–L–Y–P–V–**Dab**–L–Y–P)	Dab
5	cyclo(V–**K**–L–**K**–V–Y–P–**L**–**K**–V–**K**–L–Y–P)	GS14 (V1, K2, etc.)
8A	Ac–**K**–C–**K**–S–T–Q–D–E–Q–F–I–P–**K**–G–C–S–**K** (C–C)	03138
Helical peptides		
7	Ac–E–L–E–**K**–L–L–L–E–L–E–**K**–L–L–**K**–E–L–E–**K**–amide	LL7
7	Ac–E–L–E–**K**–L–L–V–E–L–E–**K**–L–L–**K**–E–L–E–**K**–amide	LV7
7	Ac–E–L–E–**K**–L–L–S–S–L–E–**K**–L–L–**K**–E–L–E–**K**–amide	LS7
7	Ac–E–L–E–**K**–L–L–T–T–L–E–**K**–L–L–**K**–E–L–E–**K**–amide	LT7
8B	Ac–Q–C–G–A–L–Q–**K**–Q–V–G–A–L–E–**K**–E–E–G–A–L–E–**K**–Q–V–G–A–L–Q–**K**–Q–V–G–A–L–Q–**K**–amide	01118
9, 10	Ac–**K**–I–S–A–L–**K**–E–**K**–I–S–A–L–**K**–E–**K**–I–S–A–L–**K**–E–amide	J1

[a] Peptide sequences are shown using the one-letter code for amino acid residues, except Dap denoting diaminopropionic acid and Dab denoting diaminobutyric acid; Ac = N^{α}-acetyl and amide = C^{α}-amide; potentially positively charged residues and groups are shown in **bold**.

[b] Underlined residues represent D-amino acids; lines linking N- and C-termini of linear sequences represent cyclic nature of these peptides; peptide 03138 contains an intrachain disulfide bridge, represented by line linking Cys residues.

shows the most dramatic change in column selectivity with the hydrophilic interactions now dominating the separation process, producing a much different elution profile compared to those seen at lower ACN concentrations. For instance, peptide i1 (+1 net charge) is eluted after the less hydrophilic h2 (+2 net charge); similarly, e2 (+2 net charge) is eluted after the much less hydrophilic k3 and, most dramatically, peptide l4 (+4 net charge) is eluted before the more hydrophilic c3, b3 and a3 (+3 net charge).

The flexibility of HILIC-CXC is also demonstrated in Figure 3D, where a double gradient (increasing salt gradient with concomitant decreasing ACN gradient) is employed to effect the separation, thus maintaining dominant hydrophilic (over ionic) interactions while reducing analysis time (relative to the chromatogram shown in **Figure 3**C) but retaining good column selectivity. From **Figure 3**D, this is achieved by a lower level of ACN in buffer B (50% as opposed to 90% in buffer A), which leads to a decrease in peptide retention relative to that effected by maintaining 90% acetonitrile in both mobile-phase buffers (**Figure 3**C). The peptide elution order obtained with this combined salt and ACN gradient is almost identical (save for a reversal of e2 and k3) to that shown in Figure 3C, but is obtained in about two-thirds of the time and with sharper peaks.

Although the results shown in **Figures 2** and **3** are derived from mixtures of only a limited range of peptides, they do at least provide a useful summary of the major optimization options available. Indeed, the double-gradient approach shown in **Figure 3**D is

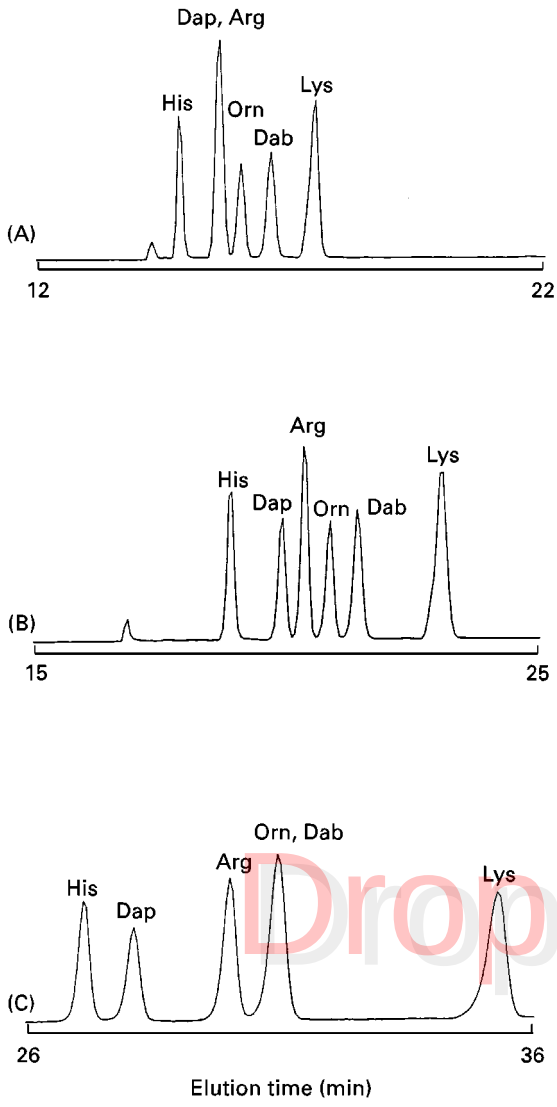

Figure 2 Effect of salt gradient steepness on HILIC-CXC of peptides. CXC column: see Figure 1. Conditions: same as HILIC-CXC conditions in Figure 1, save for 90% (v/v) acetonitrile in eluent A and 80% (v/v) acetonitrile in eluent B; linear AB gradients at (A) 5 mmol L^{-1} min^{-1}; (B) 2.5 mmol L^{-1} min^{-1}; (C) 1 mmol L^{-1} min^{-1}. (Reproduced with permission from Mant *et al.*, 1998b.)

a good starting point when first applying mixed-mode HILIC-CXC to a peptide mixture of interest. Initial run conditions similar to those described for Figure 3D, save for a lower pH (e.g. pH 3) to ensure the maximum degree of positively charged character of the peptides, are recommended. The observed elution profile can be subsequently optimized by varying gradient rate and/or mobile-phase ACN concentration as required; in addition, if acidic (potentially negatively charged) residues are present in the peptides, the pH can also be manipulated to modulate the positively charged character of the peptides and, hence, vary the strength of ionic interactions between peptides and the cation exchange matrix.

Practical Applications of HILIC-CXC for Peptide Separations

In order to appreciate fully the unique selectivity advantages of HILIC-CXC for peptide separations,

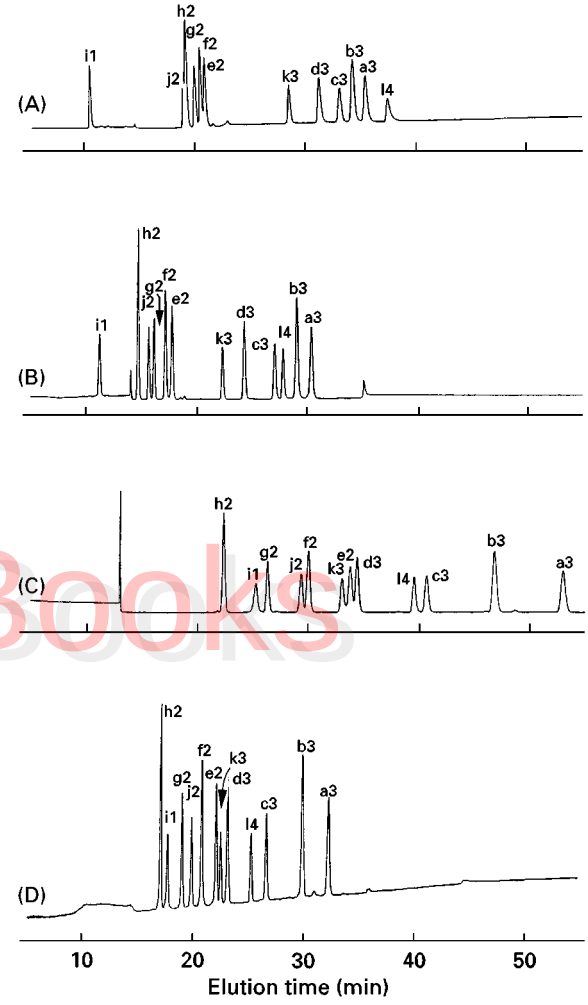

Figure 3 Effect of acetonitrile concentration on HILIC-CXC of peptides. CXC column: see Figure 1. Conditions: (panels A–C), linear AB increasing salt gradient (2% B min^{-1}, equivalent to 5 mmol L^{-1} $NaClO_4$ min^{-1}, starting with 100% A at a flow rate of 1 ml min^{-1}, where A is 5 mmol L^{-1} aqueous TEAP, pH 7, and B is A plus 0.25 mol L^{-1} $NaClO_4$, pH 7, both A and B containing 20% (panel A), 50% (panel B) or 90% (panel C) (v/v) acetonitrile; panel D, linear AB gradient) 2% B min^{-1}, equivalent to a linear increasing salt gradient of 5 mmol L^{-1} $NaClO_4$ min^{-1} and a linear decreasing acetonitrile gradient of 0.8% acetonitrile min^{-1}, starting with 100% A at a flow rate of 1 ml min^{-1}, where A is 5 mmol L^{-1} aqueous TEAP, pH 7, containing 90% (v/v) acetonitrile and B is 5 mmol L^{-1} aqueous TEAP, pH 7, containing 0.25 mol L^{-1} $NaClO_4$ and 50% (v/v) acetonitrile; all runs carried out at 26°C and peaks detected at 210 nm. (Reproduced with permission from Zhu *et al.*, 1992.)

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it is important to demonstrate the resolution of mixtures of peptides with different characteristics, e.g. random coil peptides, peptides with a defined secondary structure, constrained peptides, etc. Such a range of peptides is well represented by those presented in Table 1. In addition, the value of the HILIC-CXC is also better appreciated if its ability to resolve peptide mixtures is compared to that of the RPC mode.

HILIC-CXC is a Complementary Mode to RPC for Peptide Separations

Figure 4 compares the RPC and HILIC-CXC elution profiles of the same mixture of peptides with negligible secondary structure used in **Figure 3**. In fact, the HILIC-CXC double-gradient system at pH 7 (increasing salt gradient, decreasing ACN gradient) resulting in the elution profile shown in **Figure 3**D is here being compared to RPC at pH 7 (**Figure 4**A: including the presence of 250 mmol L^{-1} $NaClO_4$ to suppress any undesirable ionic interactions between negatively charged free silanols on the silica surface and any positively charged residues) in order to create as direct a comparison as possible between the two modes. From **Figure 4**A, the elution profile shown represents a relative measure of the hydrophobicity of the 12 peptides, from the least hydrophobic to the most hydrophobic as expressed by their increasing RPC elution times. Under HILIC-CXC conditions (**Figure 4**B), instead of a simple reversal of peptide elution, cation exchange interactions overlayed on interactions involving the hydrophilic/hydrophobic nature of the peptides now lead to useful selectivity differences between the two modes, major examples of which are denoted by arrows in **Figure 4**. The resolution of the peptide mixture is satisfactory using either RPC or HILIC-CXC, although that achieved by HILIC-CXC is superior and the complementary aspects of the two modes are quite clear.

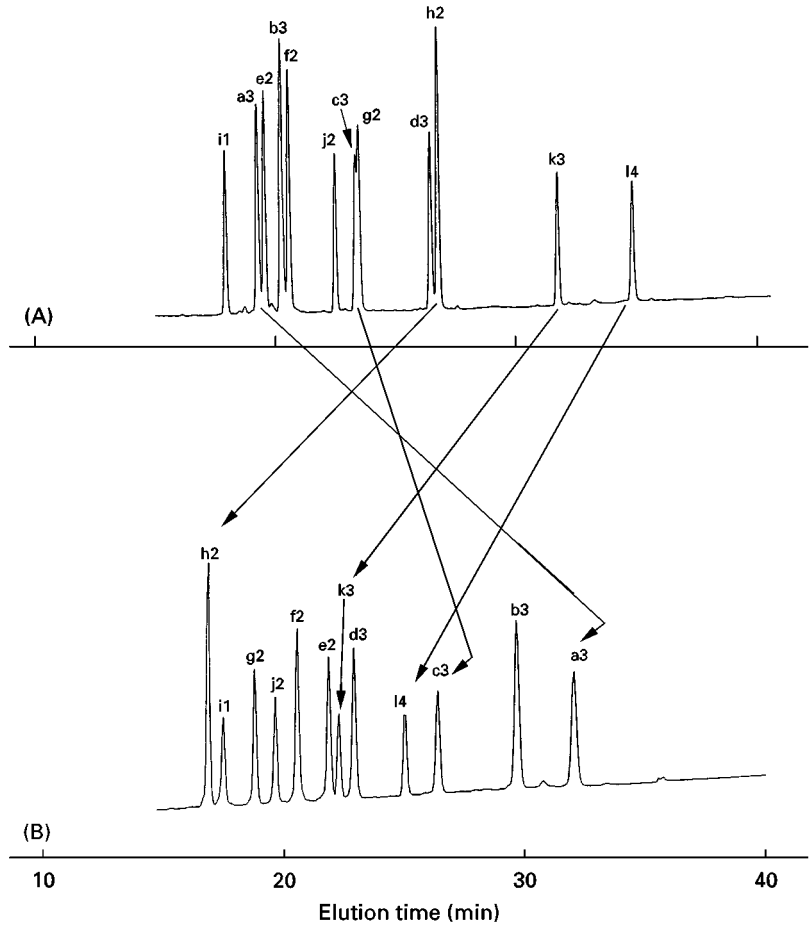

Figure 4 (A) RPC versus (B) HILIC-CXC of peptides with negligible secondary structure. Columns: see Figure 1. Conditions: RPC, linear AB gradient (1% acetonitrile min^{-1}) at a flow rate of 1 mL min^{-1}, where eluent A is 10 mmol L^{-1} aq. $(NH_4)_2HPO_4$, pH 7, and eluent B is eluent A containing 50% (v/v) acetonitrile, both eluents containing 200 mmol L^{-1} $NaClO_4$; HILIC-CXC, same conditions as Figure 3 panel D; runs carried out at 26°C and peaks detected at 210 nm. Numbers above the peptide peaks denote the number of potentially positive charges they contain. (Reproduced with permission from Mant and Hodges, 1996.)

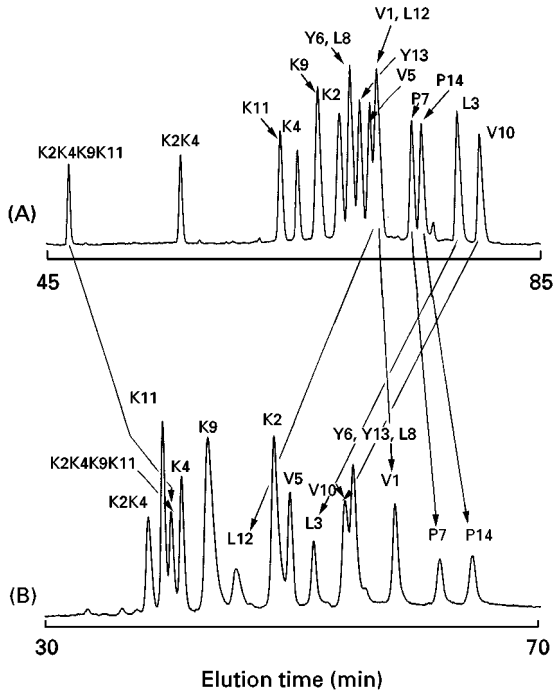

Figure 5 (A) RPC versus (B) HILIC-CXC of cyclic peptides. Columns: see Figure 4. Conditions: RPC, linear AB gradient (0.5% acetonitrile min^{-1}) at a flow rate of 1 mL min^{-1} and a temperature of 70°C, where eluent A is 0.05% aq. trifluoroacetic acid (TFA) and eluent B is 0.05% TFA in acetonitrile; HILIC-CXC, linear AB gradient 2.5 mmol L^{-1} $NaClO_4$ min^{-1}, following 5 min isocratic elution with 100% eluent A at a flow rate of 1 mL min^{-1} and a temperature of 30°C, where eluent A is 20 mmol L^{-1} TEAP, pH 3, and eluent B is eluent A containing 400 mmol L^{-1} $NaClO_4$, with eluents A and B also containing, respectively, 90% and 80% (v/v) acetonitrile. Peaks were detected at 210 nm. (Reproduced with permission from Mant *et al.*, 1998.)

Even more dramatic selectivity changes are illustrated in **Figure 5**, which compares the RPC and HILIC-CXC chromatograms for a mixture of cyclic 14-residue analogues of gramicidin S, an amphipathic β-sheet peptide. Each residue in the native GS14 sequence (Table 1) has been systematically replaced with its enantiomer, i.e. 14 diastereomers; two additional analogues are included with either double (peptide K2K4) or quadruple (peptide K2K4K9K11) L-Lys to D-Lys substitutions. The result of each enantiomeric substitution within the framework of GS14 disrupts the β-sheet structure to varying degrees, depending on the position of the substitution. Due to their isomeric nature, the 16 diastereomeric analogues have the same intrinsic hydrophobicity; thus, the differences in their RPC elution times (Figure 5A) are due to their effective hydrophobicities, i.e. the ability of a particular analogue to form a preferred hydrophobic binding domain and present this hydrophobic face to the reversed-phase matrix. From Figure 5B, the analogues with the longer retention times can present a relatively greater hydrophobic face to the reversed-phase matrix compared to analogues with low retention times which cannot present such a hydrophobic face due to more severe disruption of β-sheet structure and, hence, amphipathicity. In an analogous manner to RPC, since all 16 peptides have the same inherent hydrophilicity, their relative positions in the HILIC-CXC elution order (Figure 5B) must also be dependent on the relative disruption of β-sheet structure and amphipathicity and, hence, their effective hydrophilicities, i.e. the ability of a particular analogue to form a preferred hydrophilic binding domain and present this hydrophilic face to the ion exchange matrix.

The two elution profiles shown in Figure 5 are markedly different, reflecting profound differences in the selectivity of the two modes. Despite the complexity of the peptide elution shifts between RPC and HILIC-CXC, the more dramatic selectivity shifts (denoted by arrows) are readily apparent. Thus, peptides V1 and L12 are completely co-eluted by RPC but widely separated by HILIC-CXC; peptides L3 and V10 are the last eluted peptides during RPC, but are eluted towards the middle of the HILIC-CXC chromatogram; peptide K2K4K9K11 is eluted early during RPC, but moves significantly later in retention time relative to the other peptides during HILIC-CXC. Although both modes achieve reasonable separation of the 16-peptide mixture, this is an excellent example of where a two-column approach (e.g. HILIC-CXC followed by RPC of collected HILIC-CXC fractions), taking advantage of complementary chromatographic selectivities, is required to achieve optimal separation.

Figure 6 compares the relative effectiveness of RPC and HILIC-CXC in separating 10-residue amphipathic cyclic β-sheet analogues of gramicidin S, where substitutions have only been made on the hydrophilic face of the peptides (Table 1). Since the hydrophobic preferred binding domain of all six analogues is constant, i.e. all substitutions have been made in the hydrophilic face which tends to be oriented away from the reversed-phase stationary phase, the poor resolution of the peptides by RPC (Figure 6A) is not surprising; in contrast, these peptides are much better separated by HILIC-CXC (Figure 6B), since the preferential binding of the hydrophilic face of the peptides to the cation exchange matrix now enhances the separation. The arrows again denote relative positions of the peptide analogues between the HILIC-CXC and RPC runs in order to highlight selectivity differences between the two modes.

A similar effect of amphipathic secondary structure on the relative utility of HILIC-CXC and RPC is shown in **Figure 7**, which compares the relative

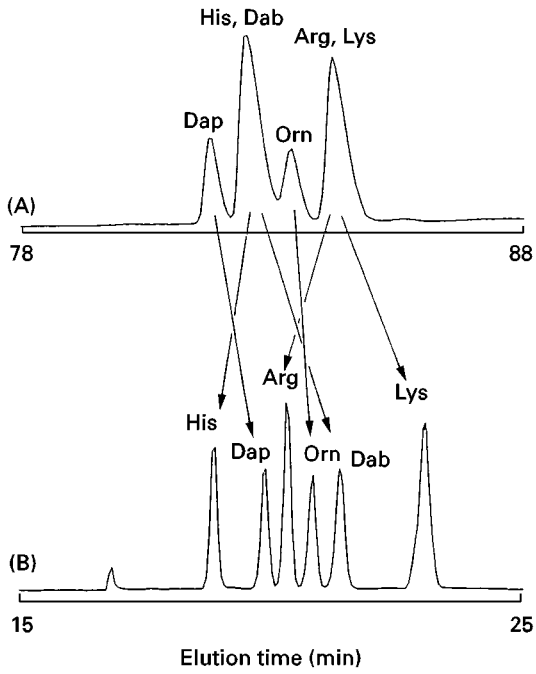

Figure 6 (A) RPC versus (B) HILIC-CXC of cyclic, amphipathic β-sheet peptides. Columns: see Figure 1. Conditions: RPC, same as Figure 1 save for 50% (v/v) acetonitrile in eluent B; HILIC-CXC, same as Figure 2 for 2.5 mmol L^{-1} $NaClO_4$ min^{-1} run. Peaks were detected at 210 nm. (Reproduced with permission from Mant *et al.*, 1998.)

effectiveness of the two modes in separating amphipathic α-helical peptide analogues LL7, LV7, LT7 and LS7 (Table 1), where substitutions have only been made in the hydrophilic face of the helices. From Figure 7A, in RPC the identical hydrophobic-preferred binding domains of the peptides bind to the hydrophobic matrix, resulting in co-elution of all four peptides under RPC conditions. Therefore substitutions in the hydrophilic face, which is oriented away from the reversed-phase matrix, have little effect on the RPC retention behaviour. In contrast to RPC, all four peptides are well resolved by HILIC-CXC (Figure 7B), even though they all have the same net positive charge, with the substitution sites in the hydrophilic faces able to interact intimately with the ion exchange matrix and, hence, influence the retention behaviour of the four analogues. Note that elution is in order of decreasing hydrophobicity, with the Leu analogue being eluted first, followed by the Val, Thr and Ser analogues, exactly as expected based on the most hydrophilic peptide being eluted last in HILIC.

HILIC-CXC for Purification and Analysis of Solid-phase Synthetic Products

In addition to RPC being the method of choice for most preparative separations of peptides, it is also commonly employed analytically to check the purity of a purified product. However, a single peak obtained during RPC is not necessarily a guarantee of peptide purity. Thus, a complementary method, such as HILIC-CXC, is required for a more accurate assessment of peak purity as well as offering an alternative approach to obtaining the required purity.

Figure 8A outlines the purification and analysis of a 35-residue cysteine-containing synthetic peptide (peptide 01118 from Table 1). The RPC elution profile of the reduced crude peptide (top) suggests a successful synthesis, i.e. a major single peak with relatively few impurities. Analysis of the subsequently RPC-purified major component by RPC (middle) shows a single, symmetrical peak. The lack of any shoulder on the peak, obtained on a very efficient column, suggests excellent peptide purity. However, mass spectrometry of this peptide showed not only the expected product mass, but a second strong signal exhibiting a peptide mass 103 units less than expected, indicating deletion of the Cys residue at position 2 of the peptide (Table 1). Although it might have been expected that deletion of a relatively hydrophobic residue such as Cys would be detected by RPC, apparently the loss of this residue has been masked by the diminishing contribution a residue

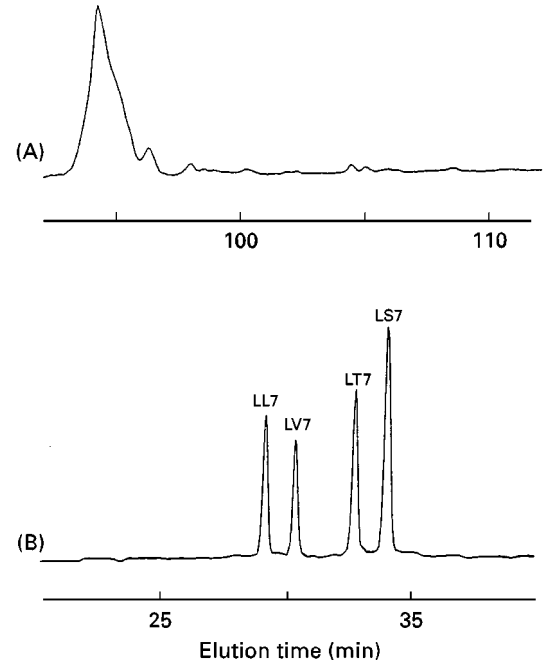

Figure 7 (A) RPC versus (B) HILIC-CXC of amphipathic α-helical peptides where substitutions have been made in the hydrophilic face. Columns: see Figure 1. Conditions: RPC, same as Figure 5; HILIC-CXC, same as HILIC-CXC run in Figure 1. Peaks were detected at 210 nm. (Reproduced with permission from Mant *et al.*, 1998.)

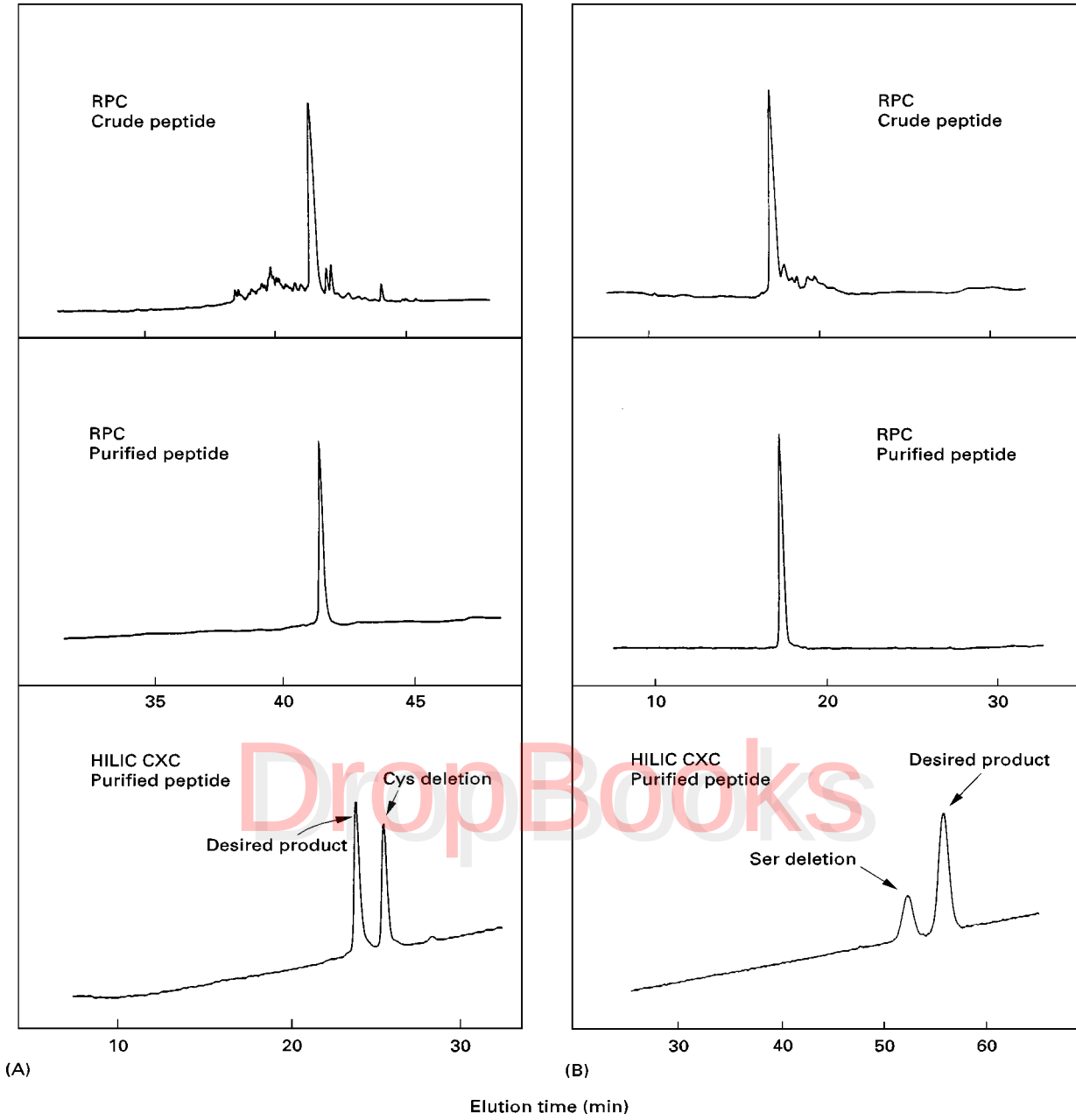

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Figure 8 Analysis and purification of synthetic peptides by RPC and HILIC-CXC. (A) 35-residue Cys-containing α-helical peptide; (B) 17-residue intrachain disulfide-bridged peptide. Column: see Figure 1. Conditions: RPC, same as Figure 5 save for a gradient rate of 1% acetonitrile min^{-1}; HILIC/CXC, linear AB gradient (2.5 mmol L^{-1} $NaClO_4$ min^{-1} from 30 mmol L^{-1} $NaClO_4$, following 10 min isocratic elution with 30 mmol L^{-1} $NaClO_4$) at a flow rate of 1 ml min^{-1} and a temperature of 26°C, where eluent A is 5 mmol L^{-1} aq. TEAP, pH 7, containing 65% (v/v) acetonitrile and eluent B is eluent A containing 400 mmol L^{-1} $NaClO_4$. Peaks were detected at 210 nm. (Reproduced with permission from Mant *et al.*, 1997.)

makes to the overall hydrophobicity/hydrophilicity with increasing peptide chain length together with any other conformation effects specific to the peptide. **Figure 8**A bottom now illustrates the excellent separation of the two peptides when HILIC-CXC is applied to the purified peptide shown in the middle RPC profile. The loss of the Cys residue makes the peptide more hydrophilic (i.e. less hydrophobic), hence the later elution of the Cys-deletion impurity. Note that neither IEC nor SEC is suitable as a complementary purification mode for this particular mixture owing to the identical net charge and essentially identical size of the two peptides. From **Figure 8**A a suitable purification protocol is HILIC-CXC of the crude peptide mixture followed by RPC of the desired product for desalting and final purification.

Figure 8B outlines the purification and analysis of a 17-residue synthetic peptide containing an intrachain disulphide bridge (peptide 03138 from Table 1). Following purification of the crude oxidized peptide mixture (top) by RPC, analysis of the purified product showed a single symmetrical peak (middle), indicating, in a similar manner to **Figure 8**A middle, excellent peptide purity. However, mass spectrometry of this single peak again showed, in addition to the expected mass, a second signal; in this case, this second signal exhibited a peptide mass 87 units less than the desired product, indicating deletion of one of the Ser residues. In RPC terms, Ser is classed as only a slightly hydrophilic group and hence contributes little to the retention behaviour of a peptide during RPC; thus, it is not surprising that the deletion product is difficult to detect, let alone resolve, by RPC. This is particularly true considering (as was subsequently determined) the position of the residue within the intrachain disulfide bridge as well as the 17-residue length of the peptide (see comments above concerning peptide chain length effects). IEC and SEC are again not suitable for isolation of the desired peptide product. However, **Figure 8**B bottom again illustrates the efficacy of the HILIC-CXC approach to resolving two peptides inseparable by RPC. Indeed, this mixed-mode approach appears to enhance the hydrophilic contribution of a Ser residue, as the Ser-deletion impurity is now baseline-resolved from the desired product. The Ser-deletion peptide is eluted prior to the desired product, since loss of this residue has made the peptide less hydrophilic (i.e. more hydrophobic) than the native peptide. In a similar fashion to the purification problem outlined in **Figure 8**A for the Cys-containing product, peptide 03138 is also best purified by an initial HILIC-CXC step followed by RPC of the desired peptide product.

Another excellent example of the utility of mixed-mode HILIC-CXC is illustrated in **Figure 9** which follows the protocol required to purify a synthetic 21-residue α-helical peptide (denoted J1 in Table 1) from the peptide crude mixture. From the RPC elution profile (top) of the crude peptide, a major peak is obtained with a number of more hydrophilic and hydrophobic impurities, some of which are eluted very close to the main component. Subsequent attempts to improve the isolation of this main peak by varying the pH and/or the ion-pairing reagent used in the RPC mobile phase were unsuccessful, thus ruling out the possibility of an efficient one-step purification by RPC. Application of mixed-mode HILIC-CXC to the crude mixture resulted in the middle elution profile which shows a major peak as well as several other smaller, yet substantial, peptide components. Collection of the major peak followed by RPC produced the bottom elution profile, with the major purified peak shown subsequently by mass spectrometry to be the desired product. Clearly, this two-step protocol was very successful in solving a difficult purification problem. Note that the middle separation, which efficient-

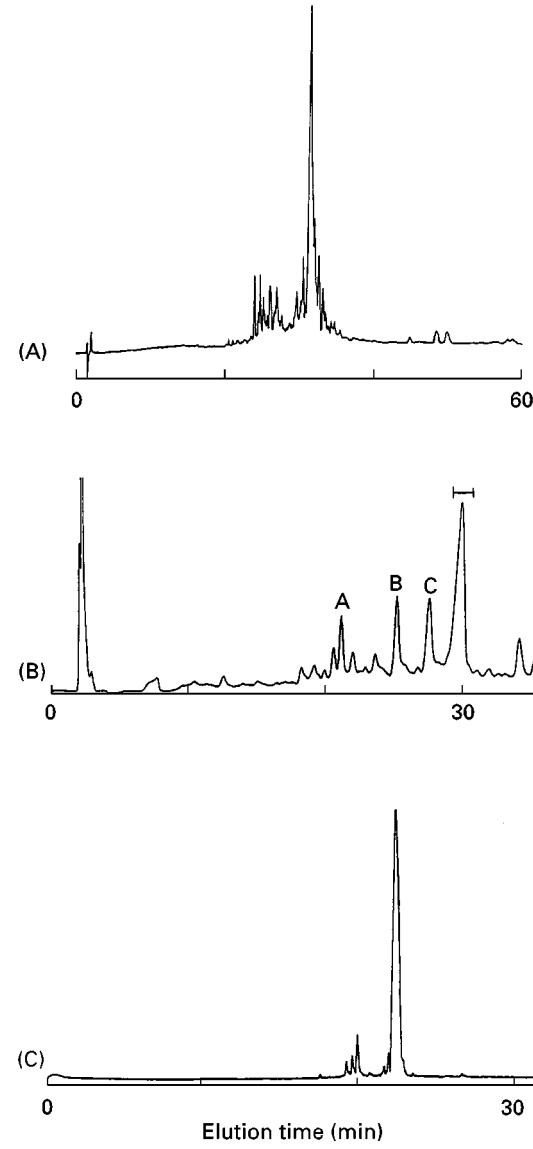

Figure 9 Two-step HILIC-CXC and RPC purification protocol for synthetic peptide. (A) RPC of crude peptide; (B) HILIC/CXC of crude peptide; (C) RPC of HILIC/CXC main peak. Columns: see Figure 1. Conditions: RPC, linear AB gradient (1% acetonitrile min^{-1}) at a flow rate of 1 mL min^{-1}, where eluent A is 0.05% aq. trifluoroacetic acid (TFA) and eluent B is 0.05% TFA in acetonitrile; HILIC-CXC, linear AB gradient 2.5 mmol L^{-1} $NaClO_4$ min^{-1}, following 10 min isocratic elution with eluent A at a flow rate of 1 mL min^{-1}, where eluent A is 10 mmol L^{-1} aq. TEAP, pH 6.5, containing 65% (v/v) acetonitrile and eluent B is eluent A containing 350 mmol L^{-1} $NaClO_4$; all runs were carried out at 30°C and peaks were detected at 210 nm. The fraction denoted by the bar in the HILIC-CXC elution profile (panel B) was collected and subsequently purified by RPC (panel C).

ly removed from the desired product the impurities eluted close to this peptide during RPC (top), could not be achieved by IEC alone, i.e. the high ACN level characteristic of HILIC-CXC was vital to the separation.

A closer look at the HILIC-CXC separation (middle profile), specifically taking note of the three major impurities (denoted A, B and C), again highlights the unique selectivity properties of this mixed-mode approach. Peak A contains Lys-deletion peptide and is thus easily resolved from the desired product due not only to the loss of a positively charged residue but also to the concomitant significant loss of hydrophilicity represented by this decrease in charge character. In contrast, peaks B and C are not deletion products, i.e. they exhibit the same net charge as the product, but are in fact peptides representing modifications of the desired peptide where random acetylation of Ser residues has occurred. Subsequent investigation identified peak B as a peptide analogue with acetylation of a single Ser at position 3 of the sequence (**Table 1**); peak C represents a peptide analogue with acetylation of a single Ser at position 10 or position 17 of the sequence or possibly a mixture of these two modified analogues. Thus, not only is HILIC-CXC able to resolve these Ser-modified analogues from the desired product, but it is also able to distinguish between the subtle differences in hydrophilicity/hydrophobicity arising from the sequence position of modification.

An explanation for the marked superiority of HILIC-CXC to resolve the Ser-modified impurities from peptide J1 may be gleaned from **Figure 10** which presents the sequence of peptide J1 as a helical net with the residues between the lines representing a wide, relatively hydrophilic face compared to the narrow hydrophobic face made up of Ile and Leu residues. The Ser residues (boxed to highlight positions of potential acetylation) lie in this wide hydrophilic face and would thus be expected to interact closely with the ion exchange matrix; conversely, they would be generally oriented away from a reversed-phase matrix, leading to a lesser effect of Ser-modification during RPC. Note that the environment (i.e. residues) surrounding Ser 10 and Ser 17 is identical while that of Ser 3 is quite different, probably accounting for the HILIC-CXC resolution of the Ser 3-modified peptide from that of analogues arising from acetylation at either of the other two positions.

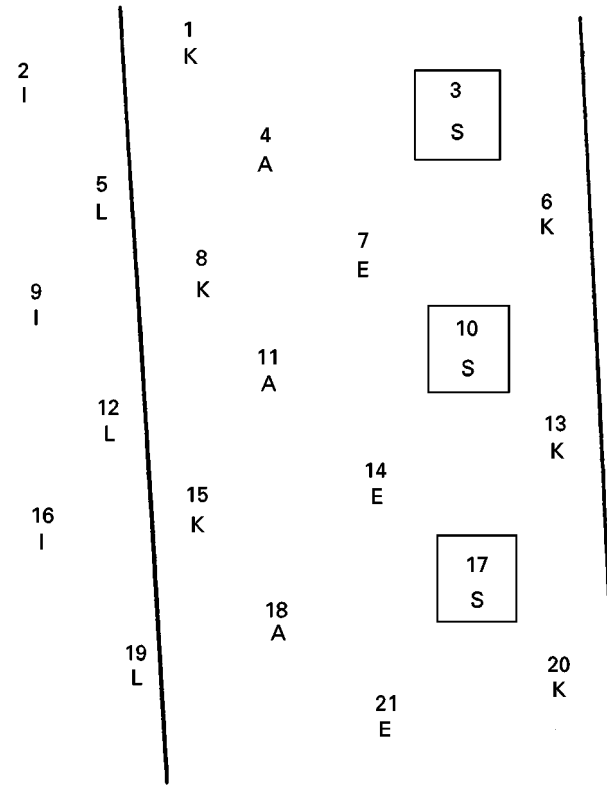

Figure 10 Sequence of synthetic amphipathic peptide J1 presented as an α-helical net. The radius of the α-helix is taken as 0.25 nm with 3.6 residues per turn, a residue translation of 0.15 nm and thus a pitch of 0.54 nm. The area between the lines represents the more hydrophilic face of the peptide, with the Leu and Ile residues representing the narrow hydrophobic face. The boxed Ser residues represent potential sites of side chain acetylation.

Future Prospects

Clearly, HILIC-CXC offers a valuable, complementary alternative to RPC for peptide separations; indeed, such an approach often rivals RPC for resolution of specific peptide mixtures. While the present article offers only a brief overview of the potential of HILIC-CXC for separation of peptides, it has already been successfully employed for protein separations where RPC alone was unable to effect the required resolution. In addition, since the need to identify peptide mixture components as well as to separate and quantify them is great, e.g. HPLC in conjunction with electrospray mass spectrometry (HPLC-MS), the utility of HILIC-CXC separations will be enhanced even further with the development of volatile mobile phases.

See also: **II / Chromatography: Liquid:** Mechanisms: Ion Chromatography; Mechanisms: Reversed Phases; Mechanisms: Size Exclusion Chromatography.

Further Reading

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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer chromatography (TLC) has found extensive application in protein chemistry including recovery of peptides in microgram and nanogram quantities for further primary structural analysis, identification of peptides in partial hydrolysates, in correlating the chromatographic properties of the intact peptides with those of individual amino acids, peptide mapping to characterize or to identify a protein available in very small quantities, resolution of diastereomeric and enantiomeric peptides without any derivatization, fractionation of proteins on the ultramicro scale, testing the optical homogeneity of synthetic peptides, and determination of relative molecular masses.

Application of TLC to the following aspects of peptide studies have also been reported: experimental studies of solute retention and support matrix effects in reversed-phase TLC (RP-TLC) of peptides; a rapid thin-layer immunochromatography method using monoclonal antibodies of two distinct specificities for quantitation of protein antigens; non-stoichiometric models for theoretical treatment of the chromatographic process on ion exchange phases; determination of amino acid configuration of synthetic peptide analogues on Chiralplate® with MeCN/MeOH/H_2O (4 : 1 : 1), prepared from the racemic aromatic amino acids; dependence of the silanophyl effect on the chemical structure of peptides and on the type of mobile phase; study of the salting out behaviour of some peptides with aromatic groups by adsorption TLC on cellulose; separation of peptides on Empore TLC sheets and blotting onto polyvinylidene difluoride (PVDF) membranes with subsequent gas-phase sequencing; analysis of peptide and protein hydrolysates by 2D cellulose TLC and densitometry and its application to luteinizing hormone.

A knowledge of the behaviour of peptides and proteins with both the mobile and stationary phases, particularly with respect to information about kinetics of diffusion, adsorption and desorption, denaturation or conformation changes, is required. Optimization of chromatographic separations of peptides and proteins means a complete resolution of all components in a minimum time, on a preparative scale and with the retention of bioactivity. Various principles of liquid chromatography have successfully been applied to TLC resolution of peptides and proteins, e.g. reversed-phase, size exclusion, ion exchange, etc. The different thin-layer materials used for the purpose include silica gel, cellulose, mixtures of silica gel and cellulose, hydroxyapatite and cross-linked dextran gel filtration media like Sephadex® (various grades form Pharmacia, Uppsala, Sweden). The ordinary porous silica-based stationary phases containing chemically bonded alkyl chains of varying lengths have several disadvantages such as low stability at alkaline pH values (pH > 8), secondary equilibria caused by low diffusion kinetics within the pores,